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Molecular Characterization of an Emerging Root-Knot Nematode *Meloidogyne enterolobii* in North Carolina, USA

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Abstract

An emerging threat to agriculture, *Meloidogyne enterolobii* Yang & Eisenback, 1983, is a tropical species and considered to be the most damaging root-knot nematode (RKN) in the world because of its wide host range, aggressiveness, and ability to overcome resistance to RKN in many crops. It was first detected in the United States on ornamental plants in Florida in 2001 but has since been identified in North Carolina, South Carolina, and Louisiana. Several thousand RKN populations were collected from North Carolina field crops, ornamental plants, and turfgrasses for species identification in the Nematode Assay Laboratory in the North Carolina Department of Agriculture & Consumer Services. From 2006 to 2019, root systems showing galling symptoms were dissected under the microscope, and females were obtained for DNA analysis. When only soil samples were submitted, the second-stage juveniles or males were used instead. Molecular characterization was performed via polymerase chain reaction with species-specific primers and DNA sequencing on the ribosomal DNA 18S-ITS1-5.8S and 28S D2/D3 and mitochondrial DNA CoxII-16S. One hundred thirty-five representative RKN populations from North Carolina were characterized and identified as *M. enterolobii.* Six populations from China where the species was originally described were included in this study for identity confirmation and comparison. As of December 2019, *M. enterolobii* has been confirmed from a limited number of fields in 11 North Carolina counties: Columbus, Craven, Greene, Harnett, Johnston, Lenoir, Nash, Pitt, Sampson, Wayne, and Wilson. Currently, *M. enterolobii* is the most important emerging RKN species in the United States and causes severe damage to agronomic and horticultural crops, especially sweetpotato in North Carolina.

Keywords: 28S D2/D3, DNA sequencing, field crops, internal transcribed spacer 1, mitochondrial DNA cytochrome oxidase gene subunit II-16S, molecular diagnosis, nematodes, pathogen detection, rDNA 18S

A root-knot nematode (RKN) population, later described as *Meloidogyne enterolobii* Yang & Eisenback, 1983, was originally described from a population that caused severe damage on the pacara earpod tree (*Enterolobium contortisiliquum* [Vell.] Morong) in Hainan Island, China in 1983 (Yang and Eisenback 1983). A few years later, a new species, *M. mayaguensis*, was described from eggplant (*Solanum melongena* L.) roots from Puerto Rico (Rammah and Hirschmann 1988). The species from Puerto Rico was later synonymized with *M. enterolobii*, based on the same esterase phenotype and mitochondrial DNA sequence (Karssen et al. 2012; Kiewnick et al. 2008; Xu et al. 2004). This species was considered an emerging, highly pathogenic RKN species. It was reported from Asia (China, India, Singapore, Tamil Nadu, Thailand, Vietnam), Africa (Benin, Burkina Faso, Congo, Ivory Coast, Kenya, Malawi, Mozambique, Niger, Nigeria, Senegal, South Africa, Togo), Central America and Caribbean (Costa Rica, Cuba, Guatemala, Guadeloupe, Martinique, Puerto Rico, Trinidad and Tobago), South America (Brazil, Venezuela), and North America (Mexico and the United States, including Florida, Louisiana, North Carolina, and South Carolina), attacking vegetables, ornamental plants, guava trees, and weeds (Brito et al. 2004; EPPO 2011, 2016; Overstreet et al. 2018; Rutter et al. 2019; Schwarz et al. 2020; Ye et al. 2013). In Europe, this species was first recorded in France (Blok et al. 2002), two greenhouses in Switzerland (Kiewnick et al. 2008), and recently in Portugal (Santos et al. 2019). Moreover, a particular concern is the ability of *M. enterolobii* to develop on crop genotypes carrying RKN resistance genes (*Mi-1*, *Mh*, *Mir1*, *N*, *Tabasco*, and *Rk*) in tobacco, tomato, soybean, potato, cowpea, sweetpotato, and cotton (Kiewnick et al. 2009). Consequently, in 2010, *M. enterolobii* was added to the European and Mediterranean Plant Protection Organization A2 Alert List and became a regulated nematode in South Korea, Costa Rica, and the United States (Arkansas, Florida, Louisiana, Mississippi, and North Carolina) (Kirkpatrick et al. 2019; Overstreet et al. 2018; USDA PCIT 2014; Wilson 2018).

Stunted cotton (*Gossypium hirsutem* cultivars PHY 375 WR and PhytoGen brand PHY 565 WR) plants with large galls visible on the roots from two separate fields near Goldsboro, Wayne County, North Carolina were collected by the first author during a field visit in December 2011. Infected plants were taken to the Nematode Assay Laboratory at North Carolina Department of Agriculture & Consumer Services (NCDA&CS) for nematode assay. Galls on cotton were larger than those commonly associated with infection by the southern RKN (*M. incognita* [Kofoid & White 1919] Chitwood, 1949). In August 2012, soybean (*Glycine max* cultivar USG 7732nRR) plants with many galls from Wayne and Johnston counties were sent to the Nematode Assay Laboratory at NCDA&CS. Microscopic examination of female perineal patterns resembled those reported for *M. incognita*. However, subsequent polymerase chain

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The author(s) declare no conflict of interest.

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Table 1. Populations of *Meloidogyne enterolobii* from North Carolina (nos. 1–135) and China (nos. 136–141) and DNA sequencing results

No.	Lab ID	County	Host	18S-ITS1	28S D23	CoxII-16S
1	12-10144	Wayne	Cotton	KP901058		MN809527
2	12-10146	Wayne	Cotton	KP901058		MN809527
3	13-633	Johnston	Soybean	KP901058		MN809527
4	13-638	Wayne	Soybean			
5	13-639	Johnston	Soybean	KP901058	KP901079	MN809527
6	13-791	Wayne	Soybean	KP901058		
7	13-6167	Johnston	Soybean			
8	14-331	Johnston	Cotton			MN809527
9	14-341	Johnston	Tobacco			MN809527
10	14-344	Wayne	Soybean	KP901058		MN809527
11	14-7068	Johnston	Cotton	KP901058		
12	14-42266	Johnston	Cotton			MN809527
13	15-4943	Johnston	Tobacco	KP901058	KP901079	
14	15-5151	Johnston	Soybean	KP901058	KP901079	
15	15-6479	Johnston	Soybean		KP901079	
16	15-6665	Johnston	Soybean	KP901058	KP901079	
17	15-20249	Wayne	Sweetpotato	KP901058	KP901079	MN809527
18	16-4159	Johnston	Sweetpotato	KP901058	KP901079	MN809527
19	16-4195	Johnston	Soybean	KP901058		
20	16-5136	Johnston	Soybean			
21	16-9950	Johnston	Cotton	KP901058	KP901079	
22	16-9951	Johnston	Sweetpotato			
23	16-12962	Johnston	Sweetpotato	KP901058		
24	16-26282	Johnston	Sweetpotato	KP901058	KP901079	MN809527
25	17-956	Johnston	Soybean	KP901058	KP901079	MN809527
26	17-3198	Johnston	Sweetpotato	KP901058	KP901079	
27	17-3646	Johnston	Sweetpotato	KP901058		MN809527
28	17-3673	Johnston	Sweetpotato	KP901058	KP901079	
29	17-4222	Johnston	Sweetpotato	KP901058		
30	17-4268	Johnston	Soybean, sweetpotato	KP901058	KP901079	
31	17-4273	Johnston	Morning glory, horseweed, sicklepod, soybean, sweetpotato	KP901058		
32	17-5572	Johnston	Morning glory, soybean, sweetpotato	KP901058	KP901079	
33	17-6400	Johnston	Sweetpotato	KP901058	KP901079	
34	17-7145	Columbus	Sweetpotato	KP901058	KP901079	MN809527
35	17-7583	Johnston	Sweetpotato	KP901058	KP901079	
36	17-8163	Johnston	Sweetpotato	KP901058		
37	17-10996	Johnston	Sweetpotato			
38	17-19585	Johnston	Sweetpotato			
39	17-23007	Johnston	Sweetpotato	KP901058	KP901079	
40	17-30534	Johnston	Sweetpotato	KP901058	KP901079	
41	17-34775	Wilson	Sweetpotato	KP901058	KP901079	MN809527
42	17-39158	Johnston	Sweetpotato			
43	17-41157	Johnston	Sweetpotato			
44	18-385	Johnston	Cotton			
45	18-1140	Johnston	Sweetpotato			
46	18-1895	Johnston	Soybean			
47	18-2796	Johnston	Soybean	KP901058		MN809527
48	18-2909	Johnston	Tobacco			
49	18-5126	Johnston	Soybean		KP901079	
50	18-6908	Wilson	Sweetpotato			
51	18-8257	Johnston	Soybean, sweetpotato			
52	18-8260	Greene	Sweetpotato	KP901058	KP901079	MN809527
53	18-8868	Johnston	Sweetpotato			
54	18-9866	Johnston	Sweetpotato			
55	18-10381	Johnston	Sweetpotato		KP901079	
56	18-11748	Harnett	Sweetpotato		KP901079	MN809527
57	18-11749	Greene	Sweetpotato			
58	18-13669	Sampson	Sweetpotato			
59	18-15251	Columbus	Sweetpotato		KP901079	MN809527
60	18-15978	Sampson	Sweetpotato		KP901079	
61	18-17926	Nash	Sweetpotato	KP901058	KP901079	MN809527
62	18-20846	Johnston	Sweetpotato		KP901079	
63	18-24354	Johnston	Sweetpotato		KP901079	
64	18-26417	Nash	Sweetpotato		KP901079	MN809527
65	18-27653	Greene	Sweetpotato		KP901079	MN809527
66	18-28969	Sampson	Sweetpotato			MN809527

(*Continued on next page*)

Table 1. (*Continued from previous page*)

No.	Lab ID	County	Host	18S-ITS1	28S D23	CoxII-16S
67	18-30046	Sampson	Sweetpotato			
68	18-30048	Greene	Sweetpotato			
69	18-33045	Sampson	Sweetpotato			
70	18-35106	Johnston	Sweetpotato			
71	18-48994	Johnston	Cucumber	KP901058	KP901079	
72	19-146	Wilson	Tobacco, soybean			
73	19-2736	Johnston	Sweetpotato			
74	19-3005	Wilson	Soybean			
75	19-3007	Wilson	Sicklepod			
76	19-4777	Wayne	Sweetpotato			
77	19-6278	Johnston	Sweetpotato			
78	19-8403	Wayne	Sweetpotato		KP901079	
79	19-8404	Wayne	Sweetpotato	KP901058	KP901079	MN809527
80	19-8721	Johnston	Soybean			
81	19-8723	Johnston	Sweetpotato			
82	19-8724	Johnston	Sweetpotato			
83	19-8725	Wayne	Sweetpotato			
84	19-8726	Johnston	Sweetpotato			
85	19-9040	Johnston	Soybean			
86	19-14008	Lenoir	Sweetpotato, soybean	KP901058	KP901079	MN809527
87	19-15731	Johnston	Sweetpotato			MN809527
88	19-19047	Nash	Sweetpotato			
89	19-22814	Martin	Sweetpotato	KP901058	KP901079	MN809527
90	19-25718	Wilson	Tobacco			
91	19-26606	Pitt	Sweetpotato			
92	19-31481	Wilson	Sweetpotato			MN809527
93	19-39725	Nash	Sweetpotato			
94	19-45671	Johnston	Soybean			
95	19-47448	Nash	Sweetpotato			
96	19-47690	Johnston	Sweetpotato			
97	19-47715	Wilson	Sweetpotato	KP901058		
98	19-48000	Johnston	Sweetpotato			
99	19-48573	Johnston	Sweetpotato			
100	19-49212	Nash	Sweetpotato			MN809527
101	19-49214	Pitt	Sweetpotato	KP901058	KP901079	
102	19-49712	Wayne	Sweetpotato			
103	19-50950	Nash	Sweetpotato, soybean	KP901058	KP901079	MN809527
104	19-51290	Wilson	Sweetpotato	KP901058		
105	19-52211	Nash	Sweetpotato	KP901058		MN809527
106	20-1060	Wilson	Sweetpotato	KP901058		
107	20-1816	Pitt	Sweetpotato, soybean			
108	20-2354	Wayne	Sweetpotato, cotton	KP901058		
109	20-2649	Nash	Sweetpotato	KP901058		
110	20-2988	Nash	Sweetpotato, cotton			
111	20-2989	Johnston	Soybean, sweetpotato			
112	20-3190	Johnston	Sweetpotato, cotton			
113	20-3483	Wayne	Sweetpotato			
114	20-3485	Johnston	Cotton		KP901079	
115	20-3486	Wayne	Soybean		KP901079	
116	20-3600	Wilson	Sweetpotato	KP901058		MN809527
117	20-5069	Johnston	Sweetpotato			
118	20-6431	Halifax	Sweetpotato		KP901079	MN809527
119	20-7186	Wilson	Sweetpotato			
120	20-11184	Johnston	Sweetpotato, soybean			
121	20-11729	Wayne	Sweetpotato		KP901079	
122	20-11731	Wayne	Sweetpotato			
123	20-11732	Wayne	Sweetpotato			
124	20-12571	Craven	Sweetpotato		KP901079	MN809527
125	20-13010	Wayne	Sweetpotato			
126	20-14345	Wilson	Sweetpotato		KP901079	
127	20-15109	Greene	Sweetpotato			
128	20-16007	Greene	Sweetpotato			
129	20-16982	Wilson	Sweetpotato			
130	20-18426	Johnston	Sweetpotato	KP901058		
131	20-20967	Johnston	Sweetpotato			
132	20-22456	Sampson	Sweetpotato			
133	20-22947	Johnston	Sweetpotato			

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Table 1. (*Continued from previous page*)

No.	Lab ID	County	Host	18S-ITS1	28S D23	CoxII-16S
134	20-22955	Johnston	Sweetpotato			
135	20-23224	Johnston	Sweetpotato			
136	991	Guangxi, China	Guava (*Psidium guajava*)	MN832683	MN832689	MN840968
137	G64	Panyu, Guangdong, China	Bitter gourd (*Momordica charantia*)	MN832684	MN832690	MN840969
138	GNc	Panyu, Guangdong, China	Weeping bottlebrush (*Callistemon viminalis*)	MN832685	MN832691	MN840970
139	GNj	Nama, Guangxi, China	Chrysanthemum (*Chrysanthemum indicum*)	MN832686	MN832692	MN840971
140	HYz	Yunlong, Hainan, China	Arrowroot (*Maranta arundinacea*)	MN832687	MN832693	MN840972
141	PYl	Panyu, Guangdong, China	Pepper (*Capsicum annuum*)	MN832688		

Table 2. Primers used in polymerase chain reaction and DNA sequencing

Primer	Amplified gene	Primer direction	Sequence (5′-3′)	Reference
SSUF07	18S	Forward	AAAGATTAAGCCATGCATG	Floyd et al. 2002
SSUR26	18S	Reverse	CATTCTTGGCAAATGCTTTCG	Floyd et al. 2002
18S965	18S	Forward	GGCGATCAGATACCGCCCTAGTT	Mullin et al. 2005
18S1573R	18S	Reverse	TACAAAGGGCAGGGACGTAAT	Mullin et al. 2005
18SnF	18S	Forward	TGGATAACTGTGGTAATTCTAGAGC	Kanzaki and Futai 2002
18SnR	18S	Reverse	TTACGACTTTTGCCCGGTTC	Kanzaki and Futai 2002
ITSUniF	18S	Forward	GTGCATGGCCGTTCTTAGTT	Ye et al. 2015
Nxy22RK5.8S	ITS	Reverse	TTCACTGCGTTCTTCATCGATC	Ye et al. 2015
ITS2.2	ITS	Reverse	CCTGGTTAGTTTCTTTTCCTCCGC	Hugall et al. 1999
D2a	28S	Forward	ACAAGTACCGTGAGGGAAAGT	Nunn 1992
D3b	28S	Reverse	TGCGAAGGAACCAGCTACTA	Nunn 1992
RK28SF	28S	Forward	CGGATAGAGTCGGCGTATC	Ye et al. 2015
28S391a	28S	Forward	AGCGGAGGAAAAGAAACTAA	Nadler and Hudspeth 1998
28S501	28S	Reverse	TCGGAAGGAACCAGCTACTA	Nadler and Hudspeth 1998
C2F3	CoxII	Forward	GGTCAATGTTCAGAAATTTGTGG	Powers and Harris 1993
1108	CoxII	Reverse	TACCTTTGACCAATCACGCT	Powers and Harris 1993
TRNAH	CoxII	Forward	TGAATTCAATCTGTTAGTGAA	Stanton et al. 1997
MRH106	CoxII	Reverse	AATTTCTAAAGACTTTTCTTAGT	Powers and Harris 1993
MeloCOIIR	CoxII	Reverse	CGATCTTTATCAGGATGAGCACC	Ye et al. 2019
Melo16SR	CoxII	Reverse	CCTTTGACCAATCACGCTAAAAGTGC	Ye et al. 2019
Me-F	IGS2	Forward	AACTTTTGTGAAAGTGCCGCTG	Long et al. 2006
Me-R	IGS2	Reverse	TCAGTTCAGGCAGGATCAACC	Long et al. 2006

reaction (PCR) and DNA sequencing on ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA) matched with *M. enterolobii*. In November 2014, *M. enterolobii* was first detected from heavily galled sweetpotato (*Ipomoea batatas* cultivar 'Covington') from Johnston County. This species of RKN has quickly become a major concern to sweetpotato growers because it affects not only the yield but also the quality of sweetpotato. Because most North Carolina sweetpotato is shipped domestically to other states or exported, it presents a quarantine problem because the nematode can be dispersed by the sweetpotato in long-distance transport, as either food or seed. This pathogen can result in total crop loss because galled sweetpotatoes are not marketable. North Carolina is the largest sweetpotato producer in the United States, and this regulated species is of great importance to the sweetpotato industry in the state. Thus, species determination of RKN is in increasing demand by farmers and regulatory agencies submitting samples to the Nematode Assay Lab at NCDA&CS.

RKN is scientifically classified in the genus *Meloidogyne* (Tylenchida: Meloidogynidae), with >100 species described so far (Ali et al. 2015; Hunt and Handoo 2009; Le et al. 2019; Tao et al. 2017). The effective use of nematode management tactics and the application of regulatory action relies on proper identification of the RKN species. Specific identification of RKN is increasingly in demand from farmers and consultants for making effective nematode management decisions such as crop rotation and plant resistance and from regulatory agencies for taking regulatory action. Unfortunately, classic morphological methods using female perineal pattern, morphology on the labial region, stylet and basal knobs on males, and morphometrics on the second-stage juveniles (Eisenback 1985) are often difficult and subjective. The differential host test (Hartman and Sasser 1985) and isozyme phenotyping of females (Esbenshade and Triantaphyllou 1985) can facilitate species identification but may not be definitive. The differential host test requires a large amount of greenhouse space and is also time consuming and labor intensive. Isozyme phenotyping is limited to the availability of active mature females, and it is difficult to get clear bands via electrophoresis. DNA-based molecular diagnosis has become a popular approach in RKN diagnosis (Phillips et al. 2005; Blok and Powers 2009; Holterman et al. 2012; Kiewnick et al. 2014; Pagan et al. 2015; Powers and Harris 1993; Powers et al. 2005; Stanton et al. 1997; Ye et al. 2015, 2019; Zijlstra et al. 2000) and is fast, sensitive, accurate, and nonsubjective and requires only a tiny amount of nematode material regardless of life stage.

Currently, 11 species of RKN have been recorded in North Carolina (Ye 2018), including *M. arenaria* (Neal, 1889) Chitwood, 1949; *M. carolinensis* Eisenback, 1982; *M. enterolobii*; *M. graminis* (Sledge & Golden, 1964) Whitehead, 1968; *M. hapla* Chitwood, 1949; *M. incognita*; *M. javanica* (Treub, 1885) Chitwood, 1949; *M. marylandi* Jepson & Golden in Jepson, 1987; *M. megatyla* Baldwin & Sasser, 1979; *M. naasi* Franklin, 1965; and *M. spatiniae* (Rau & Fassuliotis, 1965) Whitehead, 1968. Since 2006, thousands of RKN populations submitted by North Carolina growers and regulatory agencies have been analyzed through molecular diagnosis in the Nematode Assay Laboratory in NCDA&CS. The objectives of this study were to characterize 135 representative *M. enterolobii* populations through PCR by species-specific primers and DNA sequencing on the rDNA near-full-length small subunit (18S), internal transcribed spacer 1 (ITS1), partial rDNA large subunit D2/D3 (28S D2/D3), and mitochondrial cytochrome oxidase subunit II and 16S

(CoxII-16S) and to determine the species distribution in North Carolina.

Materials and Methods

Nematode samples. A total of 135 representative RKN populations positively identified as *M. enterolobii* from North Carolina were included in this study (Table 1). Some growers sent multiple samples from the same farm, but if the species was the same, only one entry was represented to minimize the sample size and duplicates. These samples were mostly submitted to the Nematode Assay Laboratory at NCDA&CS directly from growers or consultants. To confirm the identification and comparison, six populations of *M. enterolobii* were collected from Hainan, where the species was originally described, and two adjacent provinces Guangdong and Guangxi, in China. Many other RKN species were identified through this project but not included and reported in this study.

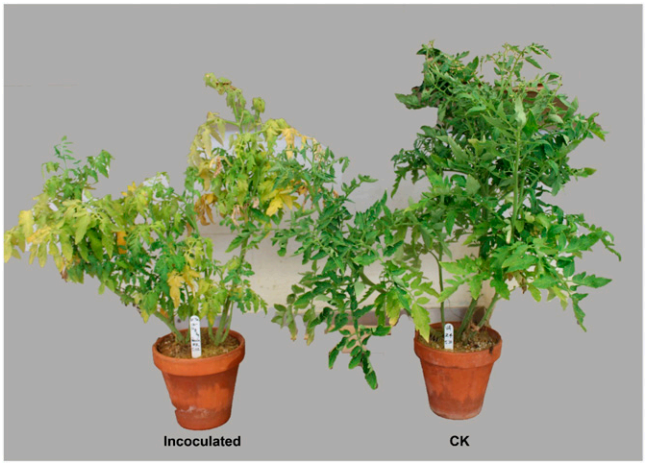

Fig. 1. Tomato foliar symptoms caused by *Meloidogyne enterolobii* (left, inoculated with *M. enterolobii*; right, not inoculated) at 90 days after inoculation (lab ID 13-633).

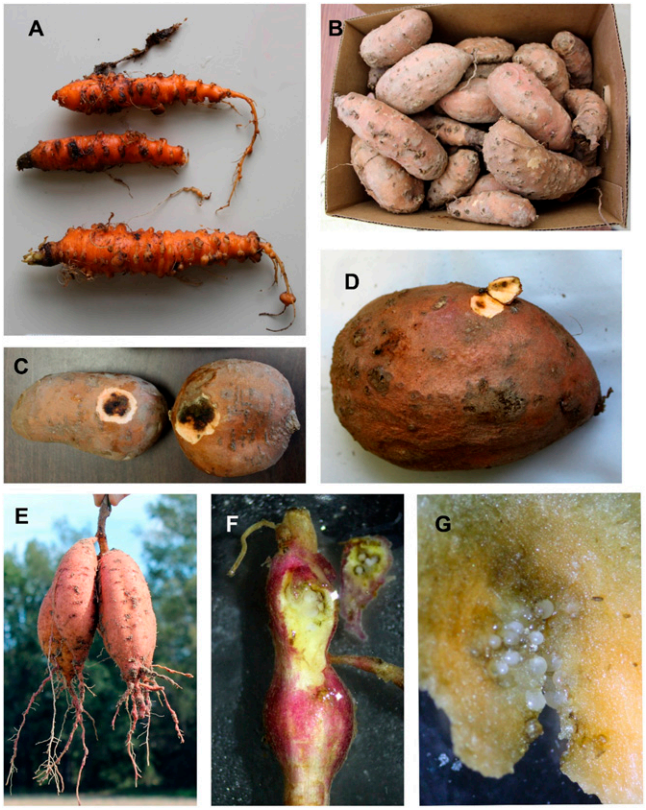

Fig. 2. Sweetpotato symptoms caused by *Meloidogyne enterolobii*. **A,** Heavily galled sweetpotato. **B,** Galled sweetpotato from packing facility. **C,** Sweetpotato from grocery store with severe lesion symptom inside. **D,** Sweetpotato from grocery store with lesion symptom inside. **E,** Freshly dug field sweetpotato plants with lightly galled tubers but severely galled fiber roots. **F,** Galled roots showing dissected females and egg masses of *M. enterolobii*. **G,** Dissected tissue from galled tuberous root showing females of *M. enterolobii*.

Extraction of nematodes. Samples submitted to the lab were soil or roots, and occasionally both. Nematodes were extracted from soil samples by a combination of elutriation (Byrd et al. 1976) and centrifugation (Jenkins 1964) methods. Galled root samples were dissected to obtain females under a Zeiss Stemi 2000-C stereo microscope (Gottingen, Germany). The nematode sample was poured into a counting dish (7.5 cm length × 3 cm width × 1.5 cm height), and the nematodes were identified and counted under a Nikon Diaphot 200 inverted microscope (Tokyo, Japan). Further morphological observation was performed with a Leica DM2500 compound microscope (Leica Microsystems Inc., Buffalo Grove, IL) with interference contrast at ≤1,000× magnification.

DNA preparation. A single female in a Petri dish filled with water after dissecting was transferred to a microscope glass slide by a pipette, crushed manually with a pipette tip, and then collected in a 1.5-ml microtube filled with 50 µl Tris-EDTA buffer (10 mM Tris-Cl, 1 mM EDTA, pH 9.0). At least three females were prepared in three separate tubes for each sample. The second-stage juveniles were prepared in the same way as females, but only one DNA template from each sample was prepared, with 1 to 10 juveniles mixed together. DNA extracts were stored at −20°C until they were used as a PCR template.

PCR by species-specific primers. The species identification of *M. enterolobii* was tested via PCR with species-specific primers Me-F and Me-R (Long et al. 2006) (Table 2). The PCR condition is the same as previously described. Three species previously confirmed as *M. incognita* (VW6), *M. javanica* (VW4), and *M. hapla* (VW9) provided by V. M. Williamson from UC Davis (Ye et al. 2015) and DNA-free water were used as negative controls.

PCR and DNA sequencing. PCR for rDNA 18S, ITS1, and 28S D2/D3 and mtDNA CoxII-16S amplifications were conducted with various combinations of universal forward and reverse primers (Table 2). The 25-µl PCR was performed with Apex *Taq* Red Master

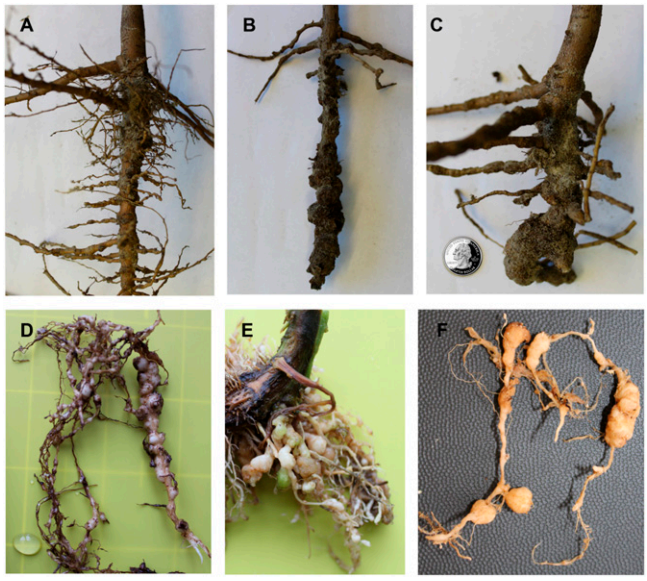

Fig. 3. Field and greenhouse photos of root galls caused by *Meloidogyne enterolobii*. **A–C,** Infested field cotton in Wayne County (lab ID 12-10144). **D,** Infested field soybean in Johnston County (13-633). **E,** Greenhouse tomato inoculated with *M. enterolobii* and harvested 90 days after inoculation (13-639). **F,** Greenhouse pepper inoculated with *M. enterolobii* and harvested 90 days after inoculation (13-639).

Mix DNA polymerase (Genesee Scientific Corporation, San Diego, CA) according to the manufacturer's protocol in an Applied Biosystems Veriti thermocycler. The thermal cycler program for PCR was as follows: denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 45 s, and extension at 72°C for 1 min. A final extension was performed at 72°C for 10 min. PCR products were cleaned with ExoSap-IT (Affymetrix, Inc., Santa Clara, CA) according to the manufacturer's protocol and were sequenced by Genomic Sciences Laboratory in North Carolina State University with an Applied Biosystems 3730 XL DNA Analyzer (Life Technologies, Carlsbad, CA). The molecular sequences were compared with other nematode species available at the GenBank sequence database in the BLASTn homology search program. The sequences were deposited into the GenBank database.

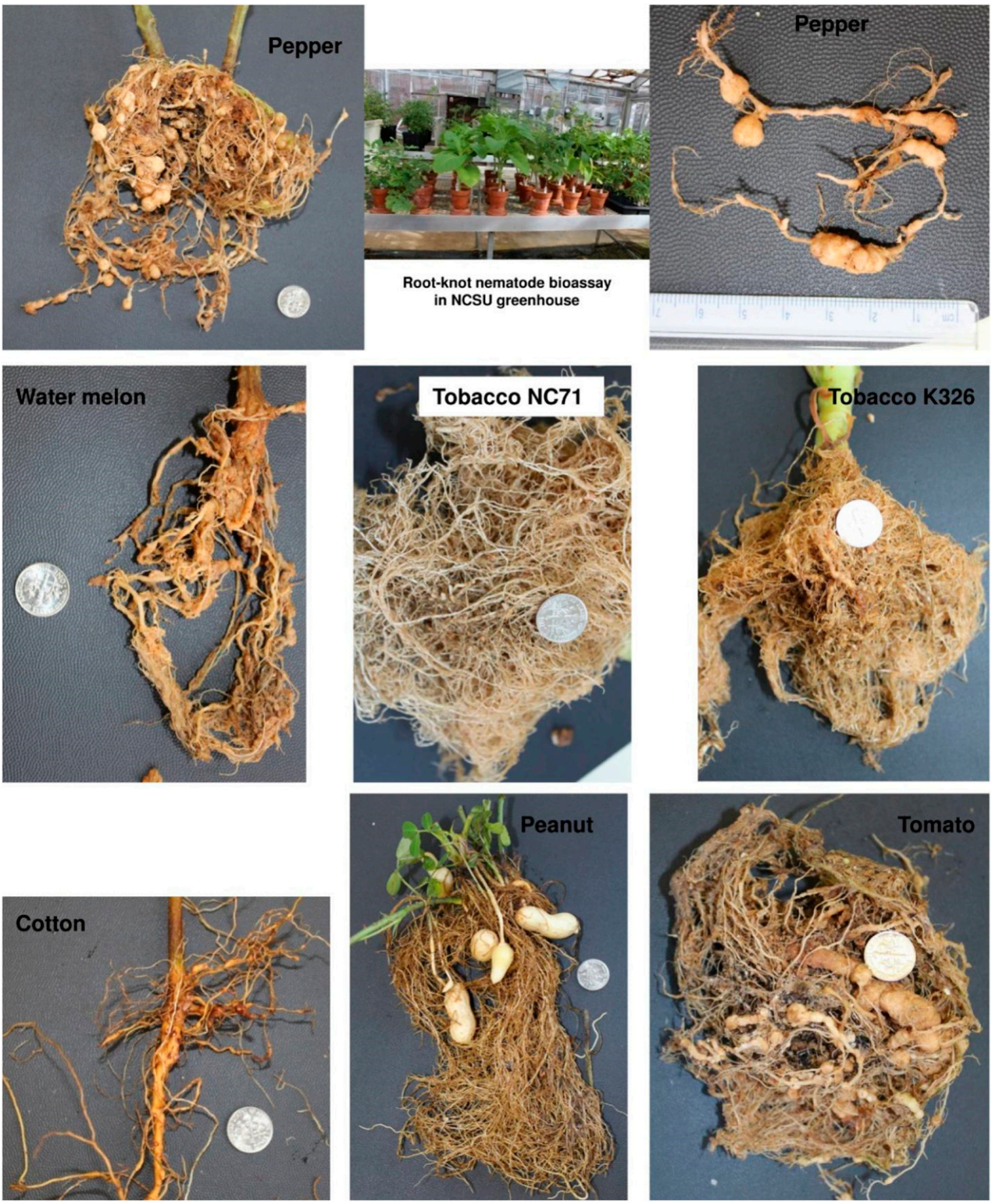

Fig. 4. Greenhouse differential host root symptoms caused by *Meloidogyne enterolobii* from a field soybean in Johnston County (lab ID 13-633). A quarter is used as a size reference.

Phylogenetic analyses. Consensus DNA sequences were edited in ChromasPro1.5 2003-2009 (Technelysium Pty Ltd, Helensvale, Australia) to obtain a contig file based on multiple forward and reverse sequences. Multiple sequences were aligned by Mega10.0.5 with default settings. The model of base substitution in the DNA sequence data were evaluated in MODELTEST version 3.06. The Akaike-supported model, the proportion of invariable sites, and the gamma distribution shape parameters and substitution rates were used in phylogenetic analyses with DNA sequence data. Bayesian analysis was performed to confirm the tree topology for each gene separately in MrBayes 3.1.0, running the chain for 1,000,000 generations and setting the burn-in at 2,500. Markov chain Monte Carlo methods were used within a Bayesian framework to estimate the posterior probabilities of the phylogenetic trees according to the 50% majority rule. The λ^2 test for homogeneity of base frequencies and phylogenetic trees was performed in PAUP* version 4.0 (Sinauer Associates, Inc. Publishers, Sunderland, MA).

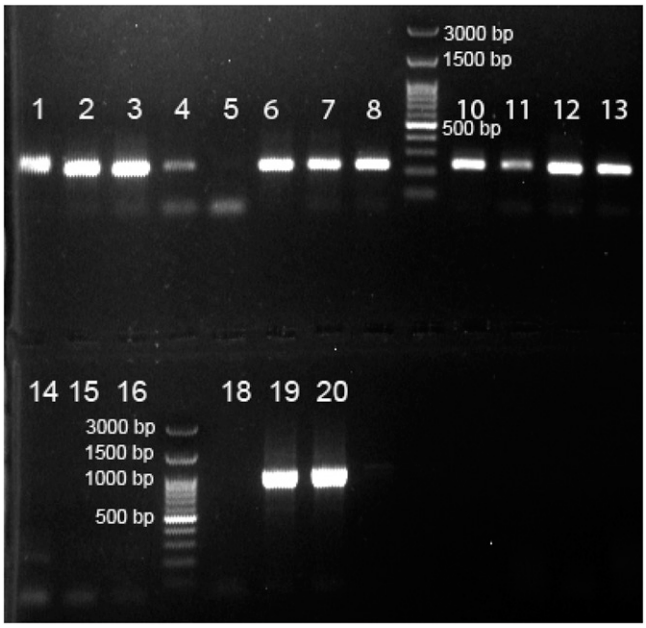

Fig. 5. Species identification via PCR with a *Meloidogyne enterolobii*-specific IGS2 primer set Me-F/Me-R (lane 1–16) and universal 28S D2/D3 primer set 28S391a/28S501 (lane 18–20). Samples were loaded on two rows in one agarose gel. Lane 1, 12-10144; lane 2, 13-633; lane 3, 13-635; lane 4, 13-639; lane 5, water; lane 6, 13-641; lane 7, 13-640; lane 8, 19-22814; lane 9, SMOBIO ExcelBand 100 bp + 3k DNA molecular ladder; lane 10, 14-336; lane 11, 19-50950; lane 12, 20-3600; lane 13, 20-18426; lane 14, VW4 (*M. javanica*); lane 15, VW6 (*M. incognita*); lane 16, VW9 (*M. hapla*); lane 17, SMOBIO ExcelBand 100 bp + 3k DNA molecular ladder; lane 18, water; lane 19, VW4; lane 20, VW6.

Results

Damage and symptoms. Damage caused by *M. enterolobii* is devastating in many crops, including cotton, soybean, pepper, tobacco, watermelon, sweetpotato, and tomato, as shown in Figures 1 to 4. Root galls are also severe on common weeds such as morning glory (*Ipomoea purpurea*), horseweed (*Conyza canadensis*), and sicklepod (*Senna obtusifolia*) in an infested soybean field. Galls on the roots are large, and the root mass and fibrous roots are scarce and small. Infected plants are usually stunted and wilted and appear nutrient deficient, as shown in Figure 1. Plants may wilt and die later in the growing season. In sweetpotato fields, aboveground symptoms are often subtle, but severe galls and bumps are very obvious on the regular roots and storage roots. When galled tissue is cut open, black lesions are often present around the RKN females (Fig. 2C, 2D, 2F, and 2G). Galled sweetpotatoes are considered poor food quality and are not marketable, which can result in total crop loss (Fig. 2A and 2B). In the field and greenhouse test, galls are common and big (Figs. 2A, 2B, 2E, 3, and 4), except for a nonhost peanut.

PCR by species-specific primers and nematode distribution and hosts. The species identification of *M. enterolobii* was confirmed via PCR with *M. enterolobii*-specific IGS2 primer set Me-F/Me-R, which produced a 236-bp DNA fragment (Fig. 5) for all samples listed in Table 1. No amplification was observed in samples VW4 (*M. javanica*), VW6 (*M. incognita*), VW9 (*M. hapla*), and water negative control (Fig. 5). Figure 5 showed about 1,000-bp PCR product for VW4 (*M. javanica*) and VW6 (*M. incognita*) but no amplification on water-negative control by universal 28S D2/D3 primer set 28S391a/28S501. The 28S D2/D3 PCR does not show size differences between RKN species (figure not shown) but shows nucleotide differences through further DNA sequencing. As of December 2019, *M. enterolobii* was confirmed from limited

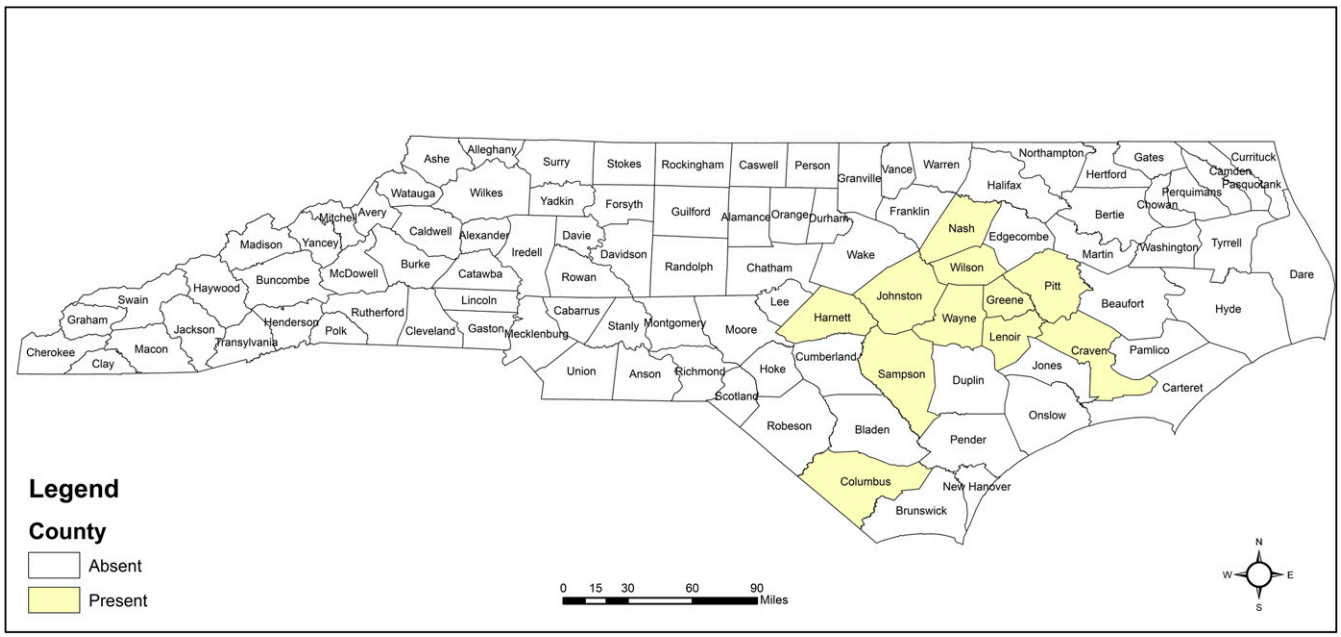

Fig. 6. Distribution of *Meloidogyne enterolobii* from North Carolina as of December 2019.

fields in Columbus, Craven, Greene, Harnett, Johnston, Lenoir, Nash, Pitt, Sampson, Wayne, and Wilson counties in North Carolina (Fig. 6). These samples are from field crops, including cotton, soybean, pepper, tobacco, watermelon, sweetpotato, cucumber, and tomato, and some common weeds such as morning glory, horseweed, and sicklepod (Table 1).

DNA sequencing. The rDNA 18S-ITS1-5.8S, 28S D2/D3, and mtDNA CoxII-16S sequences were sequenced for some of the samples and were deposited in GenBank. Their GenBank accession

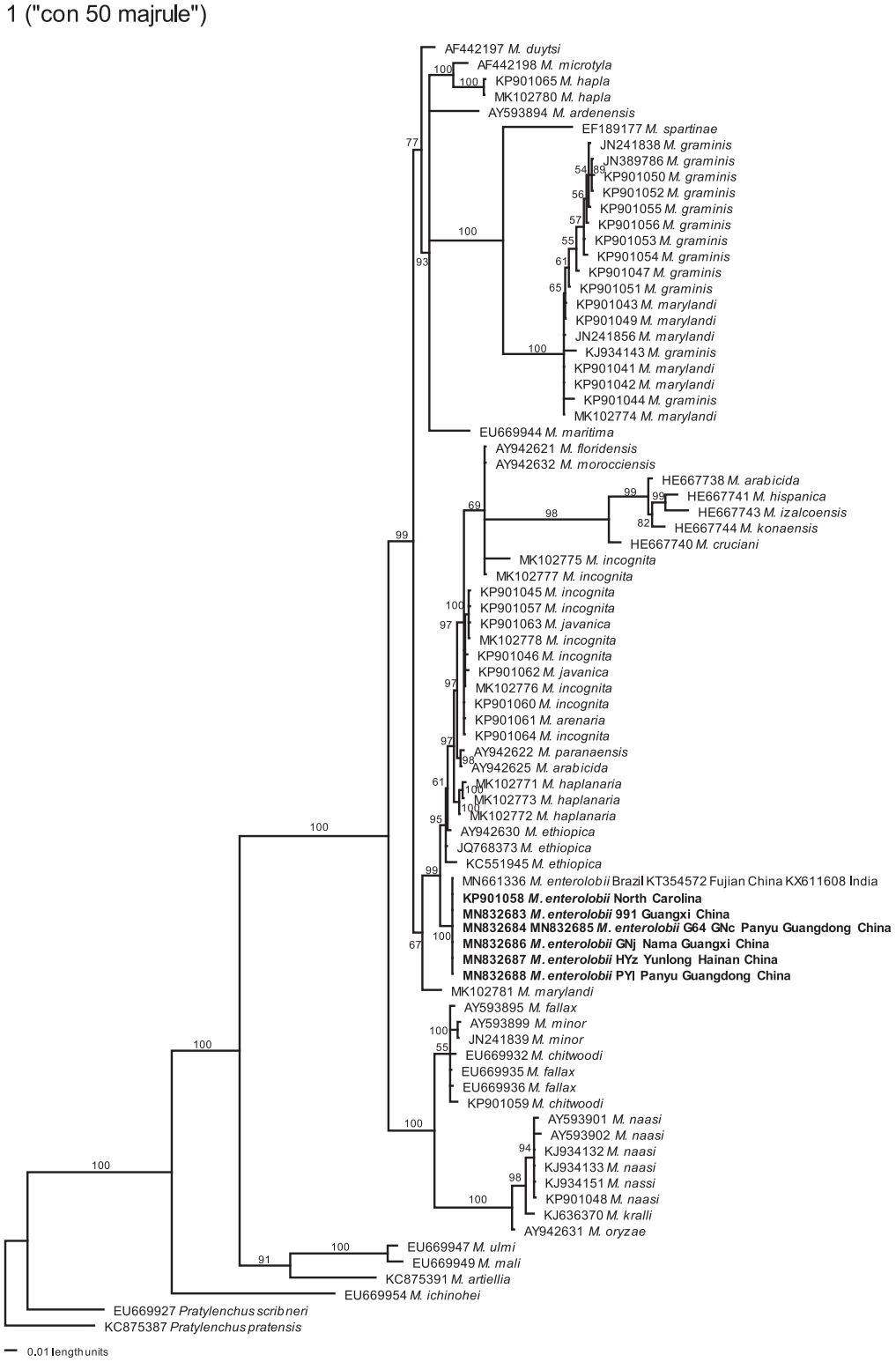

Fig. 7. Bayesian consensus tree inferred from 18S under GTR+I+G model (-lnL = 11232.8594; AIC = 22485.7188; freqA = 0.2548; freqC = 0.2152; freqG = 0.2514; freqT = 0.2787; R(a) = 1.1021; R(b) = 2.1933; R(c) = 1.6757; R(d) = 0.591; R(e) = 2.8823; R(f) = 1; Pinva = 0.4657; Shape = 0.3808). Posterior probability values >50% are given on appropriate clades.

numbers are presented in Table 1. Those sequences from different populations from North Carolina are all identical, and therefore their sequences were assigned the same accession number. Six populations from three provinces in China are also identical with those from North Carolina on three gene sequences. Blast search of these sequences matched with the sequences of *M. enterolobii* from GenBank with 99 to 100% identity.

Molecular phylogenetic relationships. A phylogenetic tree based on the rDNA 18S is presented in Figure 7, with two *Pratylenchus* species as outgroup taxa. This tree placed the North Carolina populations of *M. enterolobii* with six Chinese populations and three representative Brazilian, Chinese, and Indian populations in the same clade with 100% support. This clade is sister to another 95%-supported monophyletic clade, including some tropical RKN species

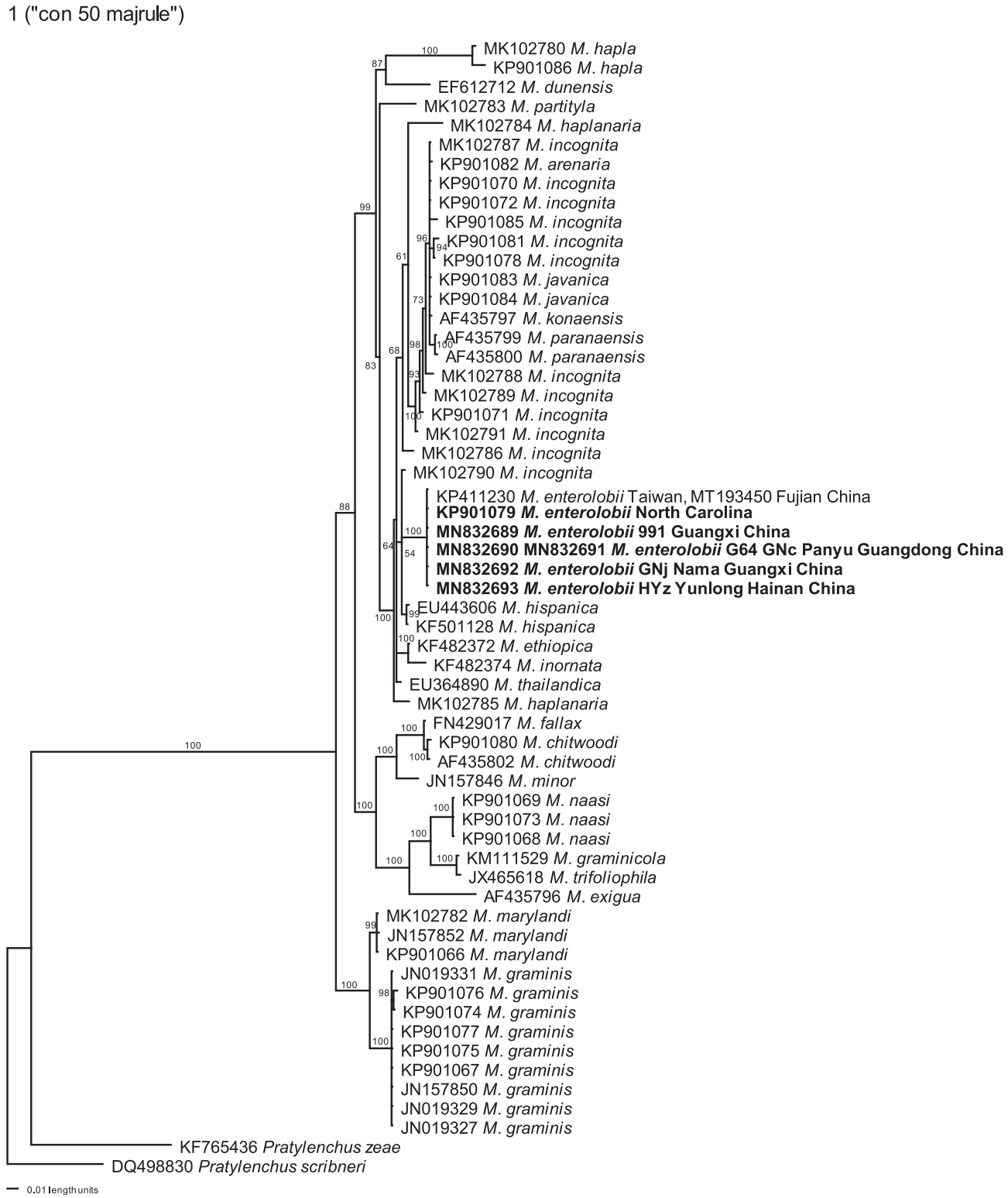

Fig. 8. Bayesian consensus tree inferred from 28S under GTR+I+G model (-lnL = 7696.4604; AIC = 15412.9209; freqA = 0.2568; freqC = 0.2149; freqG = 0.2616; freqT = 0.2667; R(a) = 1.4148; R(b) = 4.5562; R(c) = 1.6502; R(d) = 0.5011; R(e) = 7.6504; R(f) = 1; Pinva = 0.388; Shape = 0.6598). Posterior probability values >50% are given on appropriate clades.

(e.g., *M. incognita*, *M. arenaria*, and *M. javanica*) with 99% support. *M. enterolobii* is distinctly different from all other RKN species. A phylogenetic tree based on the rDNA 28S D2/D3 sequences is presented in Figure 8, with two *Pratylenchus* species as outgroup taxa. This tree placed the North Carolina populations of *M. enterolobii* with five Chinese populations and two additional Taiwan and Fujian populations in the same clade with 100% support. This clade is inside a monophyletic clade, including *M. incognita*, *M. arenaria*, *M. javanica*, *M. konaensis*, *M. paranaensis*, *M. hispanica*, *M. ethiopica*, *M. inornate*, *M. thailandica*, and *M. haplannaria* with 100% support. A phylogenetic tree based on the mtDNA CoxII-16S sequences is presented in Figure 9, rooted with *M. partityla* (MK102796), which is based on the multiple-sequence alignment whose sequence is most distinct from the other sequences. No outgroup species in another

Fig. 9. Bayesian consensus tree inferred from CoxII-16S under TVM+G model (-lnL = 4746.0107; AIC = 9508.0215; freqA = 0.3414; freqC = 0.0405; freqG = 0.1166; freqT = 0.5015; R(a) = 2.5374; R(b) = 5.884; R(c) = 1.164; R(d) = 2.099; R(e) = 5.884; R(f) = 1; Pinva = 0; Shape = 0.7745). Posterior probability values >50% are given on appropriate clades.

genus was included in the analysis because of the excessive sequence divergency in this gene. The mtDNA sequence among RKN species is much more divergent, as seen in a comparison of rDNA 18S and 28S long branch length between Figure 9 and Figures 7 and 8. This tree placed the North Carolina populations of *M. enterolobii* with five Chinese populations and other *M. enterolobii* populations in the same clade with 100% support. This clade is sister to six populations of *M. haplanaria* with 100% support but with a long branch length difference. The mtDNA sequence of *M. enterolobii* is very different from that of all other RKN species, including *M. incognita*, *M. arenaria*, and *M. javanica*.

Discussion

Since the first report of *M. enterolobii* detected from North Carolina (Ye et al. 2013), thousands of samples have been submitted to the Nematode Assay Laboratory at NCDA&CS to determine the RKN species. Rapid and accurate species identification has become a serious challenge for the lab. This study characterized the introduced species through PCR with species-specific primers and DNA sequencing on the rDNA 18S-ITS1-5.8S and 28S D2/D3 and mtDNA CoxII-16S. One hundred thirty-five representative RKN populations from North Carolina were characterized and identified as *M. enterolobii.* Six populations from China where the species was originally described were included in this study for comparison and showed no difference with North Carolina populations. As of December 2019, *M. enterolobii* was confirmed from limited fields in 11 North Carolina counties: Columbus, Craven, Greene, Harnett, Johnston, Lenoir, Nash, Pitt, Sampson, Wayne, and Wilson. The emergence of *M. enterolobii* as a pest new to North Carolina is problematic because it appears to be spreading within the state. This research makes it possible to conduct a more accurate survey of RKN populations in North Carolina, which is needed from both a management and regulatory perspective. A systematic survey is essential to determine the distribution and extent of the *M. enterlobii* infestation at both the state and national level.

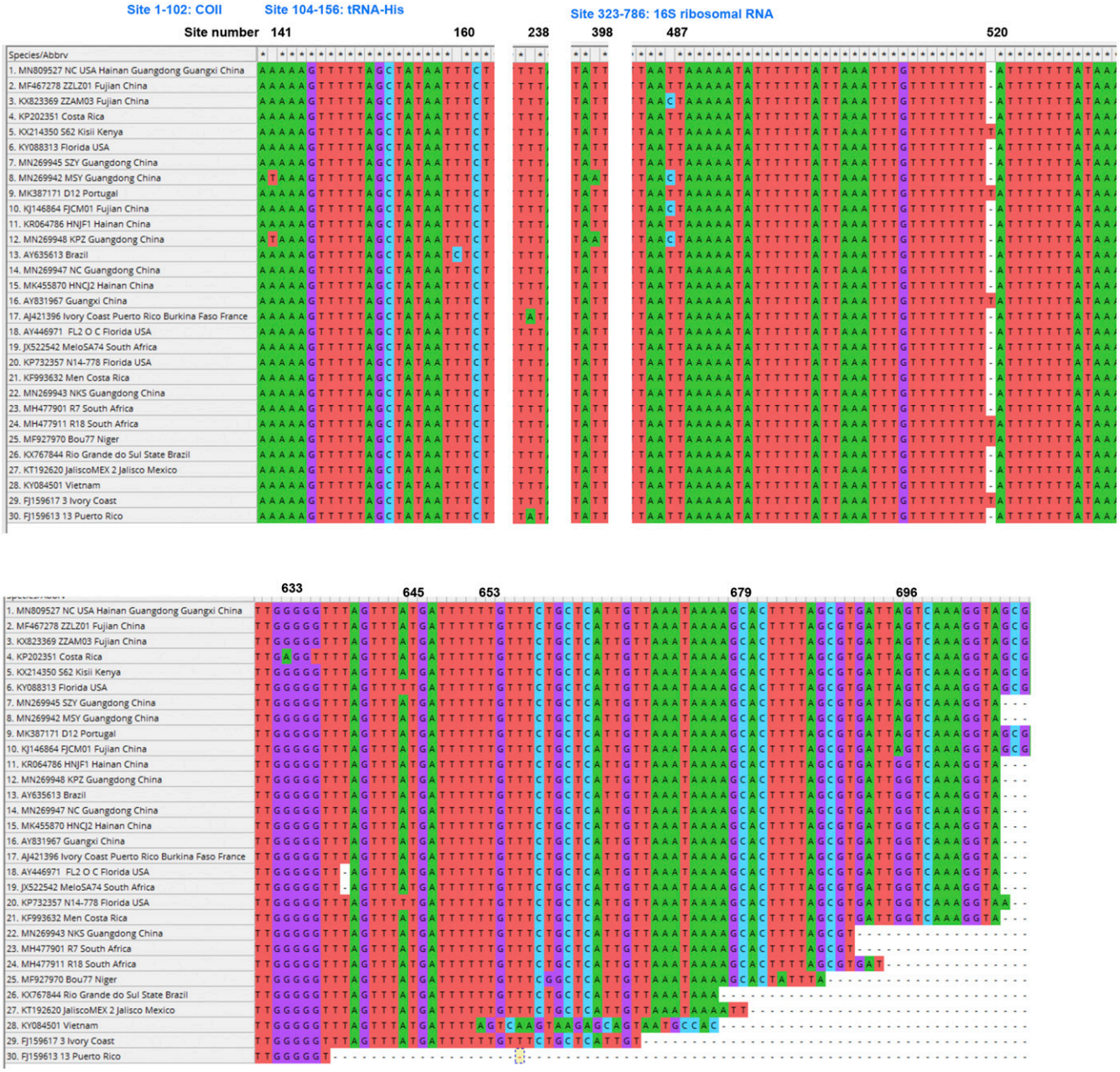

Fig. 10. Portions of multiple DNA sequence alignment of CoxII-16S of representative populations of *Meloidogyne enterolobii* from around the world. Total characters are 786; most conserved sites not shown.

In this study, the species identification of *M. enterolobii* was successfully accomplished via PCR with *M. enterolobii*-specific IGS2 primer set Me-F/Me-R, which produced a 236-bp DNA fragment (Long et al. 2006) for all samples in Table 1. These samples were either a single female or the second-stage juveniles, occasionally male, but no significant difference was observed in PCR between life stages. This primer set is robust and sensitive, and no false positives were observed because most of the results were further confirmed by DNA sequencing on three genes. As a result of this research, this approach has been adopted as the standard for the Nematode Assay Laboratory at NCDA&CS as a service to the growers. PCR by the universal primers listed in Table 2 is not always successful, and in general, CoxII-16S is more difficult to amplify (data not shown). Changing various combinations of forward and reverse primers is necessary to ensure more success in PCR. Reasons for the samples in Table 1 lacking sequencing results include the following: No PCR or DNA sequencing was attempted after positive identification by *M. enterolobii*-specific primers; PCR failed; PCR was successful, but DNA sequencing failed; or both PCR and DNA sequencing were successful, but other microorganism or host plant genetic material was also amplified and sequenced. From this study, the DNA sequence of *M. enterolobii* was revealed to be specific on all three genes and is distinctly different from all other species, particularly CoxII-16S. However, DNA sequencing takes additional effort, time, and resources and is usually performed by another DNA sequencing lab; thus, it is not suited for a large-scale, routine assay but is a reliable approach to verify the results of PCR with species-specific primers.

Phylogenetic analysis on three genes of *M. enterolobii* revealed that this species is closely related to tropical RKN species (e.g., *M. incognita*; *M. arenaria*; *M. javanica*; *M. haplanaria* Eisenback, Bernard, Starr, Lee & Tomaszewski, 2003; *M. lopezi* Humphreys-Pereira, Flores-Chaves, Gomez, Salazar, Gomez-Alpizar & Elling, 2014; and *M. ethiopica* Whitehead, 1968), as observed in many other studies (García and Sanchez-Puerta 2015; Holterman et al. 2009, 2012; Humphreys-Pereira et al. 2014; Kiewnick et al. 2014; Powers et al. 2018; Ye et al. 2019). Based on the more divergent mitochondrial CoxII-16S sequence, this species is much closer to *M. haplanaria* (Texas peanut RKN). This finding is also supported by Santos et al. (2019) based on CoxI and CoxII-16S. *M. haplanaria* was originally found attacking peanut in Texas (Eisenback et al. 2003) and was also reported from Arkansas (Khanal et al. 2016; Ye et al. 2019) and *Mi*-resistant tomato in Florida (Joseph et al. 2016). Based on current limited available data, *M. enterolobii* probably evolved from the same origin with *M. haplanaria* in evolution within the genus of *Meloidogyne*, but this supposition must be tested in the future as more species are described and sequenced.

CoxII-16S is a valuable marker to differentiate RKN species. The PCR amplicons by C2F3/1108 (Powers & Harris 1993; Powers et al. 2005) differ in size between many RKN species, such as *M. chitwoodi*; *M. hapla*; *M. exigua* Göldi, 1887 (about 500 bp); *M. enterolobii* (about 700 bp); *M. arenaria*; *M. floridensis* Handoo, Nyczepir, Esmenjaud, van der Beek, Castagnone-Sereno, Carta, Skantar and Higgins, 2004 (about 1,100 bp); *M. paranensis* Carneiro, Carneiro, Abrantes, Santos & Almeida, 1996 (about 1,250 bp); *M. lopezi* (about 1,370 bp); *M. arabicida* Lopez & Salazar, 1989; *M. incognita*; *M. izalcoensis* Carneiro, Almeida, Gomes & Hernandez, 2005; and *M. javanica* (1,500 to 1,680 bp) (Humphreys-Pereira et al. 2014; Powers et al. 2005; Smith et al. 2015). However, rDNA 18S and 28S do not have these size variations between RKN species and are more conserved. About 90 CoxII-16S sequences are available for *M. enterolobii* from GenBank to examine the DNA sequence divergence among world populations compared with our North Carolina and China populations. These sequences of *M. enterolobii* are distinctly different from those of all other known RKN species. Figure 10 presents multiple sequence alignments from 30 representative sequences. This alignment has 786 characters including a partial 3′ region of CoxII (site 1 to 102), transfer RNA-histone (site 104 to 156), and a partial 5′ region of 16S rRNA (site 323 to 786). Most of the conserved sites were not shown in this alignment. There is no variation in CoxII, one substitution in site 141 at transfer RNA-histone, and two insertions/deletions in site 520 and 639 and 34 insertions/deletions in the other regions, especially at 16S rRNA toward the 3′ end. In general, Sanger DNA sequencing can provide about 550 bp high-quality sequences, and the first 50 bp and any sequences after 550 bp have poor quality, which should be trimmed off when making contig file. All sites, particularly variable sites, should be carefully examined for quality before deposit to GenBank. Based on our large raw DNA sequencing data from North Carolina and China, no sequence variations were observed, and they are identical to some other populations from elsewhere in the world in GenBank. In Figure 10, sequences from site 633 to 709 are beyond the good-quality region of Sanger sequencing, and thus those variations are probably poor-quality sites and should be removed, such as the last sequences of AGTCAAGTAAGAGCAGTAATGCCAC from KY084501, TT from KT192620, A from KP732357, and the last fifth A from MF927970. Six sequences in site 520 have an insertion, which is a common error to read a poly T region as either eight or nine Ts. Eleven sequences on site 696 with G are also at the very end of the sequences and could be A like the other sequences. All the other variable sites represent only one or a few sequences and should be carefully examined to represent the true differences. When the obvious errors are removed from site 653 to 786, our sequence (MN809527) is identical to FJ159617 from Ivory Coast; KT192620 from Mexico; KX214350 from Kisii, Kenya; KX767844 from Rio Grande do Sul State, Brazil; KY084501 from Vietnam; MF467278 from Fujian, China; MH477901 from South Africa; MH477911 from South Africa; MK387171 from Portugal; MN269943 from Guangdong, China; and MN269945 from Guangdong, China. The remaining variable sites need further testing, and they are probably all identical.

M. enterolobii is extensively distributed in regions with typical tropical climatic conditions, including Asia, Africa, South and Central America, and the Caribbean. It has also been first reported from areas of North America exhibiting a warmer climate, such as Florida (Brito et al. 2004). This study revealed that this species can become well established in a colder region, as indicated by its occurrence in 11 counties in North Carolina where the winter temperature is commonly below freezing. North Carolina appears to be the most northern region for this tropical species at this time in the United States, and it poses a major threat to this state's agricultural economy because sweetpotato is the number one crop produced in the state.

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